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Original Article

Triplex Real-time Polymerase Chain Reaction Optimization for AZF Y-chromosome Microdeletion Analysis

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ABSTRACT

Objective: Y chromosome microdeletions at the "Azoospermia Factor" regions (AZFa, AZFb, AZFc) are the second genetic cause of spermatogenic failure in infertile men. Despite its importance for the treatment of infertile patients, no prior investigations have been previously published in Ecuador. . The purpose of this study is to optimize a molecular technique that allows detection of microdeletions in the AZF region.

Methods: Using a genomic DNA of healthy male with natural conceived offsprings, a multiplex real time polymerase chain reaction (qPCR) was standarized with eigth sequence-tagged site (STS) sY85, G34990, sY133, sY127, sY254, sY255, and using as internal control sex-determine region Y (SRY) and Ameologenin Y (AMELY). With this technique, 35 DNA samples taken from peripheral blood of patients with severe oligozoospermia were analyzed.

Results: A triplex qPCR was standardized using EvaGreen DNA-binding dye to obtain melting temperature (Tm) of the STS previously mentioned. Three of the patients evaluated were detected to have partial microdeletion in the AZFa region, with a frequency of 8.8%; being losses in the G34990 section (one patient) and sY85 section (two patients). No cases of microdeletions in other AZF regions were found.

Conclusion: The triplex qPCR optimizated allows the identification of microdeletions in AZFa, AZFb and AZFc region in infertile men and a better clinical management of the patient's treatment decision. This first report for Ecuador reveled a higher prevalence of microdeletions in the AZFa region in comparison with those previously described in other populations.

Keywords: AZF, Microdeletion, PCR, Y Chromosome, Male infertility.

INTRODUCTION

Infertility affects about 15% of couples worldwide, 50% of these cases due to male factors (Elfateh *et al.*, 2014; Kovac *et al*., 2013; Sheikhha *et al*., 2013; O'Flynn *et al.*, 2010; Kozina *et al*., 2011; Dohle *et al*., 2010).

Within the etiology of male infertility, the genetic factor plays an important role, influencing in a variety of physiological processes such as spermatogenesis and sperm quality. This factor would represent between 15 to 30% of the causes of male infertility (Elfateh *et al*., 2014; Khabour *et al*., 2014; O'Flynn *et al.*, 2010; Dohle *et al.*, 2010; Kleiman et al., 2012), being microdeletions of the Y chromosome (YCMD) the second genetic cause of spermatogenic failure in infertile men, after Klinefelter syndrome (Khabour *et al*., 2014; Kozina *et al*., 2011; Guo *et al.*, 2012; Silber, 2011).

In the Y chromosome, there are three regions referred to as "Azoospermia Factor" (AZFa, AZFb, AZFc), all related to spermatogenesis and normally altered or lost in YCMD, this is found with a frequency of 13% in men with non-obstructive azoospermia and 7-10% in men with oligozoospermia (Yuen *et al.*, 2014; Zaimy *et al*., 2013; Kozina *et al.*, 2011;. Guo *et al*., 2012; O'Flynn *et al*., 2010; Soares *et al*., 2012).

The AZFa region contains two major genes involved in fertility, these are USPY and DBY. The total deletion of this region is rare (5%) and only a few patients have been described. It is associated with the complete absence of germ cells, presenting Sertoli cells-only syndrome (SCO) (Sheikhha *et al*., 2013; Krausz *et al*., 2014; Suganthi *et al.*, 2013; Silber, 2011; Kleiman *et al,* 2012; O'Flynn *et al*., 2010). In these cases it is impossible to retrieve sperm from the testes for assisted reproduction treatments (ART) such as intracytoplasmic sperm injection (ICSI) (Guo *et al*., 2012).

Partial deletions of AZFa have a wider range of prevalence (0.2%-11%) and its histological characteristics range from normozoospermia to SCO (Kleiman *et al*., 2012), depending on the clinical syndrome, the man may or may have not have problems in producing offspring.

In the AZFb region several genes are found, being the main RBMY and PRY. Deletions in this area are more common (10%-16%) than those of the AZFa region, nevertheless these are found in a very low percentage of azoospermic men (Krausz *et al*., 2014; Suganthi *et al*., 2014; Silber, 2011; Soares *et al*., 2012). The total deletion of the region completely stops spermatogenesis, causing azoospermia and total absence of sperm in the testicles. This would imply not have sperm through testicular sperm extraction (TESE) process (O'Flynn *et al*., 2010; Silber, 2011).

Rolf *et al.* (2002) have reported the presence of mature sperm cells with deletions in the region AZFb, associating microdeletions in such area with moderate oligozoospermia, allowing natural conception and therefore the transmission of this genetic abnormality. It is possible to find sperm cells in men with partial deletions in this region (Krausz *et al*., 2014; Suganthi *et al*., 2013; Kleiman *et al*., 2011).

Deletions in AZFc region are the most frequent (50% and 60%) (Kleiman *et al*., 2011; Soares *et al*., 2012; Silber, 2011). They produce a wide range of phenotypes associated with low sperm concentration and reduced spermatogenesis, being found both in azoospermic or severely oligozoospermic men (O'Flynn *et al*., 2010; Guo *et al*., 2012; Navarro-Costa *et al*., 2007). This suggests that genes located in this area (mainly the DAZ family) are involved in the maturation of post meiotic germ cells or sperm (Soares *et al*., 2012; Silber, 2011).

For men who are azoospermic with deletions in the AZFc area, it is possible to obtain sperm through TESE and to conceive by ICSI, considering that Y chromosome genetic disorders would be transmitted to the male offspring (Kleiman *et al.*, 2011; Guo *et al*., 2012).

The diagnosis of any YCMD can clarify the cause of idiopathic non-obstructive azoospermia or oligozoospermia, influencing over the treatment options (Kozina *et al*., 2011; Dohle *et al.*, 2010; Guo *et al.*, 2012).

Current methods for diagnostic are based on poly-

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Table 1. Description of the used primers.

Name	Primers	Concentration (iM)	Amplicon Tm (ºC)	Amplicon Size (bP)
SRY (Control)	GAATATTCCCGCTCTCCGGA/ GCTGGTGCTCCATTCTTGAG	125	86.5–86.68	470
AMELy (Control)	ATCAGAGCTTAAACTGGG-AAGCTG/ CTCTGTAAAGAA- TAGTGGGTGGAT	500	78.9-79.3	105
sY85 (AZFa)	GCTATTCTCTCTCTGGCATC-TGTATT/ TGGCAATTTGCCTAT- GAAGT	750	76.5-77.0	80
G34990 (AZFa)	CATTCGGTTTTATCAGCCAG/ CAGTGACTCGAGGTTCAATG	1500	76.4-76.7	83
sY133 (AZFb)	ATTTCTCTGCCCTTCACCAG/ TGATGATTGCCTAAAGGGAA	500	83.4-84	177
sY127 (AZFb)	GGCTCACAAACGAAAAGAAA/ CTGCAGGCAGTAATAAGGGA	300	81.9-82.2	274
sY255 (AZFc, DAZ1-4)	GTTACAGGATTCGGCGTGAT/ CTCGTCATGTGCAGCCAC	125	84.3-84.5	124
sY254 (AZFc, DAZ1-4)	GGGTGTTACCAGAAGGCAAA/ GAACCGTATCTACCAAAGCAGC	150	81.4 – 82.0	380

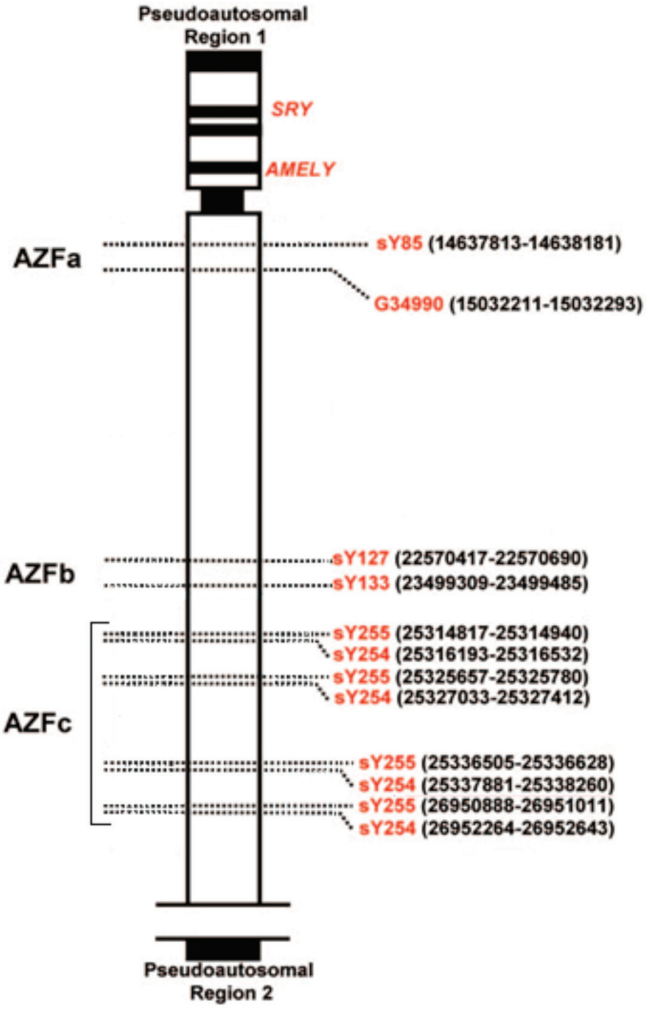
Table 2. Primers combination in the Duplex and Triplex qPCR.

DUPLEX qPCR		TRIPLEX qPCR	
Reaction	Primers	Reaction	Primers
A	SRY (Control) sY254 (AZFc, DAZ1-4)	A	SRY (Control) sY85 (AZFa) sY127 (AZFb)
B	sY85 (AZFa) sY133 (AZFb)	B	SRY (Control) sY133 (AZFb) sY254 (AZFc, DAZ1-4)
C	AMELy (Control) sY255 (AZFc, DAZ1-4)	C	AMELy (Control) G34990 (AZFa) sY255 (AZFc, DAZ1-4)
D	G34990 (AZFa) sY127 (AZFb)		

Table 3. Oligozoospermic patients with AZFa microdeletion.

Affected Section	G34990	sY85
# Patients with partial microdeletion	1	2
Incidence of microdeletion	2,9%	5.9%
AZFa microdeletion frequency	8.8%	

Figure 1. Location of the Primers used for the multiplex PCR, in the Y chromosome. Modified scheme from Kozina *et al*. (2011). On the left side the AZFa, AZFb, AZFc regions can be identified. On the right the union sites for the primers are identified in red and their genomic position is shown in black.



merase chain reaction (PCR), amplifying specific sequence sites (STS) within the region of interest. The European Academy of Andrology (EAA) and European Network of Molecular Genetics Quality (EMQN) published the guide for the molecular diagnosis of Y chromosome microdeletions (Krausz *et al.*, 2014; Kozina *et al*., 2011; Guo *et al*., 2012; Simoni, 2004, 2001).

Despite the importance of the evaluation of this type of microdeletions in infertile men, no prior investigation has been published in Ecuador about this topic.

The aim of this estudy is to optimize a molecular technique that allows detection of microdeletions in the AZF region.

MATERIAL AND METHODS

Patients

Peripheral blood samples from 35 oligozoospermic men without offspring were obtained from the National Center of Assisted Reproduction Innaifest. For Positive control, peripheral blood samples from two fertile men (25 and 23 years old) with naturally conceived children, were considered. The semen parameters were evaluated acording WHO (2010).

Isolation of DNA

Genomic DNA were extracted using High Pure PCR Template Preparation Kit, version 16.0 (Roche: Cat. No. 11 796 828 001) according to the manufacturer's protocol. The concentration of extracted DNA was determined by measuring the UV absorbance at 260 nm with the "Biophotometer" spectrophotometer (Eppendorf).

Primers

Eight primer pairs were setected for the publication of Kozina *et al.* (2011) (Figure 1). Two pairs were used as internal control and six primer pairs were specific to the sites AZFa, AZFb and AZFc (Table 1).

Multiplex Real-Time PCR

The study was carried out in three triplex real-time PCR using the Mastercycler, Version 2 Channels 520 nm / 550 nm (Eppendorf). PCR conditions were 95ºC for 60 seconds, followed by 35 cycles of 95ºC for 15 seconds, 60ºC for 45 seconds, 72ºC for 30 seconds and a final cooling of 60°C for 30 seconds. The 20 ul reaction contained 1X EvaGreen® SsoFast™ (Bio-Rad). The concentration of primer was listed in Table 1.

Melting Analysis

The melting curve was run after cycling by a ramping from 60ªc to 95ºC (with a temperature transfer speed of 0.02ºC/second).

RESULTS

Using the DNA of healthy controls, the amplification conditions and melting temperatures (Tm) of each segment of interest was determined (Table 1). Then, we combined different primers performing reactions with 2 (duplex) and 3 (triplex) sets of primers (Table 2), identifying the Tm of each product, in both multiplex qPCR (Figure 2 and 3).

Triplex qPCR products of control samples were analyzed on an 2% agarose gel, obtained distinguishable bands in each of the three reactions (Figure 4), allowing to correlate and confirm the results of dissociation temperature.

The sensitivity of the Triplex qPCR was evaluated by using DNA from a positive control and conducting the PCR with subsequent melting analisys on increasing four-fold dilutions. The mealting peaks could be accurately identified up to an initial DNA concentration of 2.47 ng / ml., after 35 cycles of amplification.

We attempt to develop a quadruplex qPCR, however it was not possible to display the 4 products with sufficient discrimination between each Tm.

The evaluation of the 35 DNA samples with the triplex qPCR established found 3 patients with partial microdeletions in AZFa region (Figure 5) and none microdeletions in other regions investigated (AZFb and AZFc), having an incidence of the AZFa microdeletion of 8.8% (Table 3).

DISCUSSION

For development the multiplex real time PCR (qPCR), each set of primers were evaluated with DNA control of fertility men. The intercalating fluorescent agent –EvaGreen- was used to optimize the Tm of each PCR product.

With duplex qPCR, Tm of each product was identify, but with a reduced amplification efficiency in sY85 (AZFa), G34990 (AZFa) and sY254 (AZFc, DAZ1-4) sequences (Figure 3). The problem was solved by changing the concentration of the three sets of primers.

Using the optimized parameters in the duplex qPCR,

Figure 2. Melting Curves of the Duplex qPCR. The chart shows the melting temperatures of each set of primers when combined, also a deficency of the sY85, sY254 y G34990 primers is shown.

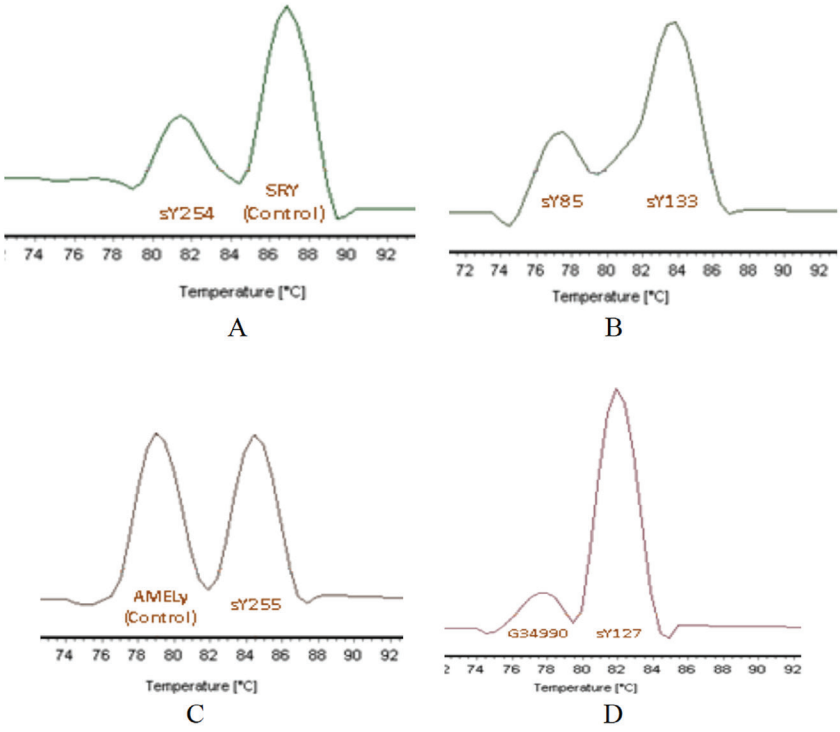


Figure 3. Melting Curves of the Triplex qPCR. The chart shows the differentiation of the melting curves of each set of primers when combined.

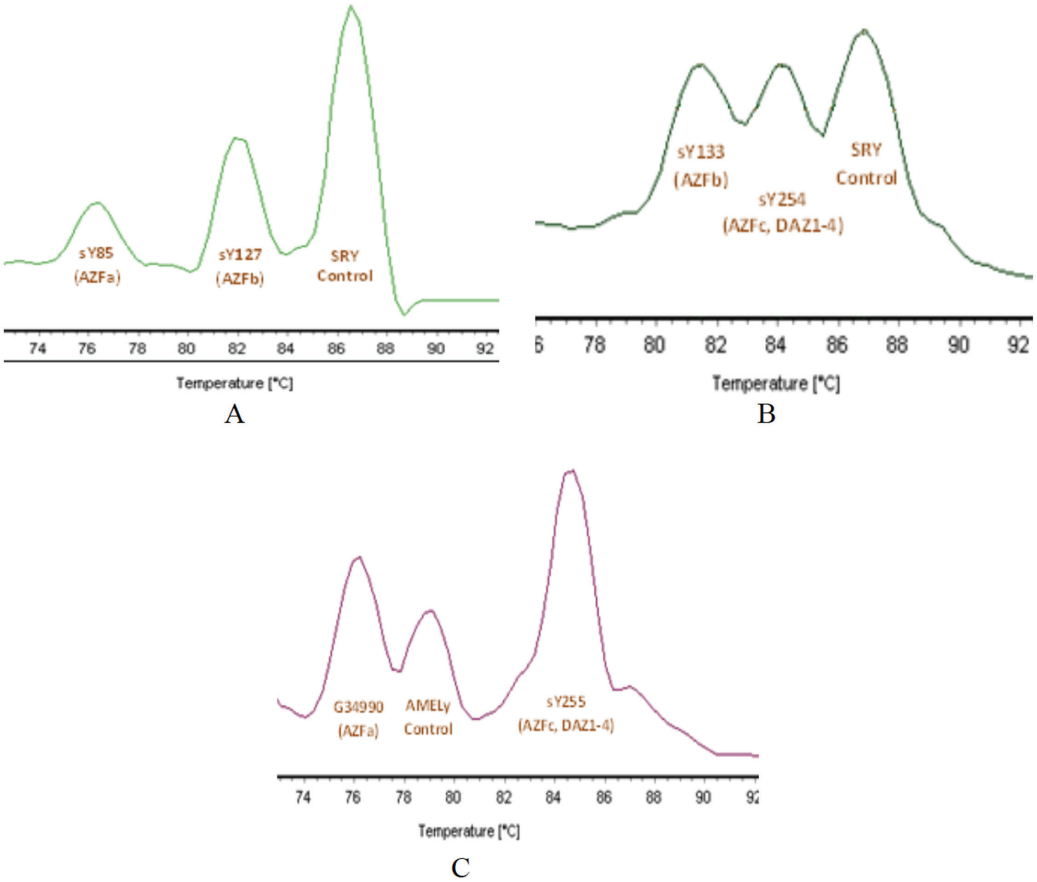


Figure 4. Observation of control samples amplicons in an electrophoresis gel. On the right side, the sequence names are describe and on the left, their weight is displayed.

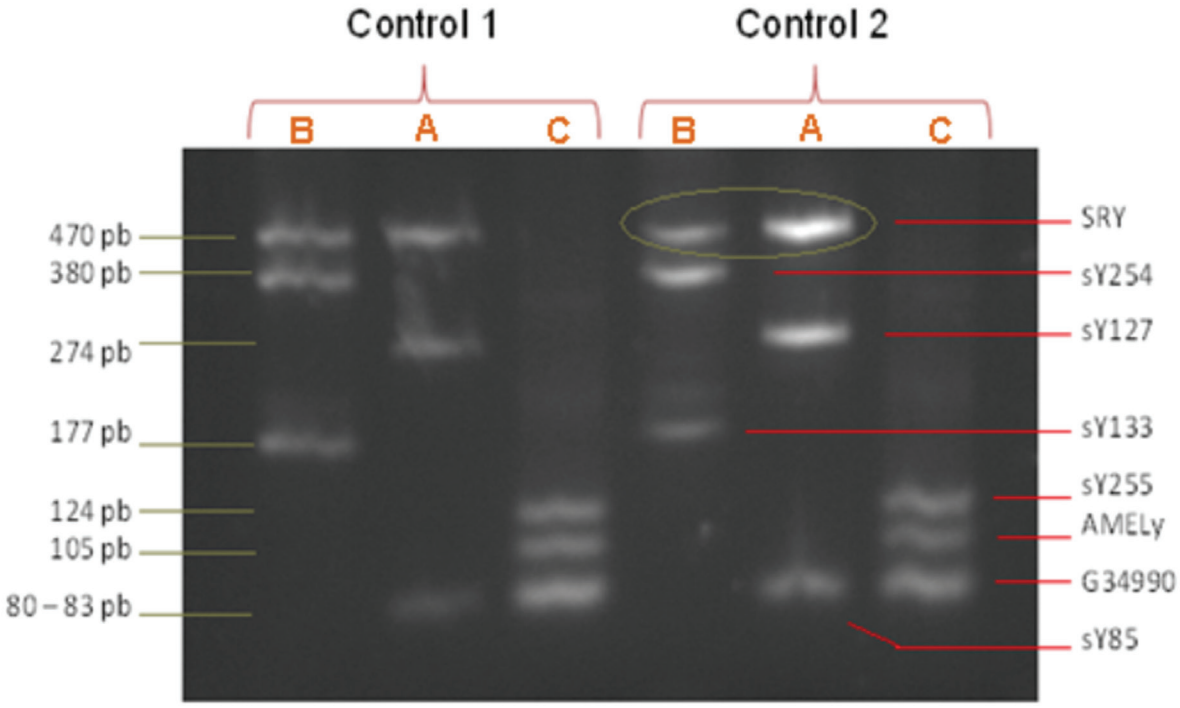
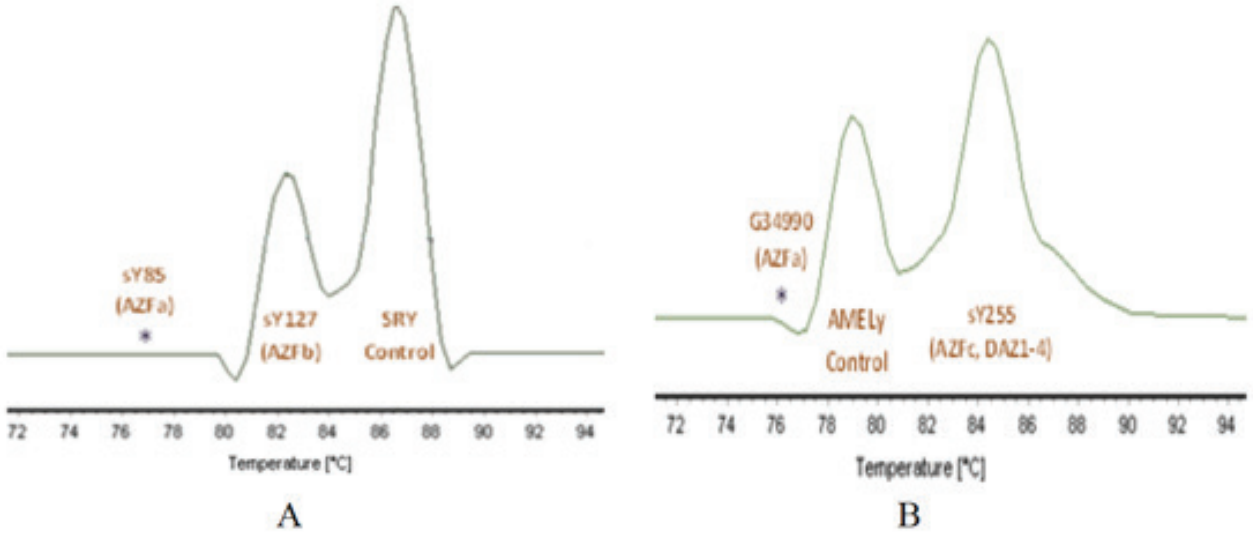


Figure 5. Microdeletions identified on the samples. Figure A and B show absence of sY85 and G34990 regions in the triplex qPCR.



we proceeded to test a triplex qPCR, combining primers as indicated in Table 1. Differentiable dissociation curves for each amplicon were obtained (Figure 4).

We try to establish a quadruplex qPCR, combining the primers as described by Kozina *et al.* (2011); however it was not possible to display the four products with sufficient discrimination between each temperature. This might give to the poor sensitivity of the thermocycler used.

Triplex qPCR sensitivity identifies accurate melting temperature with 2.47 ng/ml DNA initial concentration, after 35 amplification cycles. This sensitivity is lower than thareported by Guo *et al.* (2012) and Kozina *et al.* (2011); nevertheless, Guo *et al.* (2012) working with probes that give higher sensitivity and specificity than a DNA-binding dye such as EvaGreen.

The 35 oligozoospermic cases evaluated with optimized technique found three patients with partial AZFa microdeletions, corresponding to 8.8% incidence (Table 3). No case with microdeletions in AZFb and AZFc was found.

These results reveal a higher incidence than that reported in other studies such as Silber (2011), Kleiman *et al.* (2012), Behulova *et al.* (2010) and O'Flynn *et al.* (2010); nevertheless, like Fu *et al.* (2012), this study found only partial microdeletions and not total deletions. AZFa partial

deletions prevalence have a wide range (0.2% -11%) (Kleiman *et al.* 2012), not defined by the few studies reported.

Studies report a AZFc microdeletions as the most frequent. The variation of the results found in this study could be a result of the geographic localization and racial characteristics of the studied population; however, to corroborate theses preliminary findings, it would be necessary to expand the number of samples.

The work regarding the incidence of AZF microdeletions have been mainly conducted on Asian and European populations. There are few studies published in Latin America as Lucena *et al.* (2004) and Fernández-Salgado *et al.* (2006), both reporting deletions in the AZFc region with a frequency of 6% and 3.4% respectively; but like this study, the number of cases included in both publications (97 and 29 respectively) was very low to determine the incidence.

Other genetic alterations involved in male infertility have been identified (Kozina *et al*., 2011; Buch *et al*., 2003; Kovac *et al*., 2013). This designed multiplex technique is restricted for identifying large deletions in the AZF section, like Kozina *et al.* (2011) and Simoni *et al*.(2001) protocols, it may be possible that the evaluated patients present other genetic problems in the Y chromosome that can not be identified with this technique.

This work would be the first Ecuadorian report about the optimization of a molecular technique to detect microdeletion in AZF region of Y chromosome.

CONCLUSIONS

The triplex qPCR optimizated allows the identification of microdeletions in AZFa, AZFb and AZFc region in infertile men and a better clinical management of the patient's treatment decision. This report, done for the first in Ecuador, reveled a higher prevalence of microdeletion in the AZFa region in comparison with those previously described in other populations.

CONFLICT OF INTERESTS

No conflict of interest have been declared.

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